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T Cell Responses to BPV-4 E7 during Infection and Mapping of T Cell Epitopes

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Vaccination of cattle with the recombinant E7 protein of bovine papillomavirus type 4 (BPV-4) prior to BPV-4 infection has been shown to retard development of papillomas and accelerate their regression. To understand the mechanism of regression we have measured proliferation of peripheral blood mononuclear cells (PBM) to E7 *in vitro* during the course of BPV-4 infection in both vaccinated and nonvaccinated cattle. In vaccinated cattle, T cells specific for E7 could be detected at high levels shortly after challenge, whereas in nonvaccinated cattle low responses of E7-specific T cells could be detected in only a few animals at the late stages of papilloma development. Using short overlapping synthetic peptides corresponding to the E7 protein, three T cell epitopes have been identified. T1 (aa 31–59) was immunodominant and T2 (aa 70–88) and T3 (aa21–40) were minor epitopes. © 1995 Academic Press, Inc.

INTRODUCTION

Papillomaviruses can infect and cause proliferative lesions in both cutaneous and mucous epithelia. The virus-induced tumours are normally benign and regress spontaneously, with a few exceptions. Human papillomavirus type 16 (HPV-16) has been frequently implicated with squamous cell carcinoma of the cervix (zur Hausen, 1991) and BPV-4 with alimentary canal cancer in cattle (Campo, 1992). Given its association with cancer, it follows that a vaccine against the virus either preventing infection or controlling preexisting lesions at an early stage would prevent development of cancer. The immune response to papillomaviruses is still unclear but an understanding of the immunology will be helpful in the development of a vaccine. Circumstantial evidence has implicated cell mediated immunity in the control of papillomavirus infection. Individuals with primary or secondary cellular immunodeficiency have persistent infections (Benton *et al.*, 1992). Other evidence for the importance of a cellular immune response to papillomavirus is the observation of infiltration of lymphocytes into regressing BPV-2-induced papillomas of cattle (Jarrett *et al.*, 1991), in cottontail rabbit papillomavirus lesions (Okabayashi *et al.*, 1993), and in flat warts in humans (Tagami *et al.*, 1974; Berman and Winkelman, 1977). The possibility of direct experimentation in animal systems allows vaccination studies and the analysis of the immune response of a host to its natural pathogen over time. BPV-4 infection of the alimentary canal in cattle leads to the development of papillomas which can progress to cancer in the presence of cofactors such as feeding on bracken fern, which contains immunosuppressants (Evans *et al.*, 1982a) and mutagens (Evans *et al.*, 1982b). We have previously reported that vaccinating cattle with recombinant E7 of BPV-4 retards tumour growth and accelerates regression of BPV-4-induced papillomas (Campo *et al.*, 1993). E7 specific antibodies have been detected in the sera of vaccinated animals and three immunodominant B cell epitopes have been identified on the protein (Chandrachud *et al.*, 1994). This paper extends the study of the immune response to E7 in control (nonvaccinated) and vaccinated cattle by analysing the response of T cells to E7 protein and mapping T cell epitopes.

MATERIALS AND METHODS

β-Galactosidase E7 fusion proteins

The early E7 open reading frame (ORF) was cloned in pUR or pGEX plasmids to produce β-galactosidase (βGal) or glutathione–S-transferase (GST) fusion products, respectively, as previously described by Campo *et al.* (1993). Briefly, the E7 ORF, representing full-length E7, was isolated as a *Bsr*I fragment [nucleotides (nt) 652–1249] and cloned into pUR278 by the addition of *Bam*HI linkers or into pGEX3x by blunt end cloning into the *Sma* site. The recombinant was transfected into *Escherichia coli* JM109, grown and induced by IPTG. βGal, GST, and the corresponding E7 fusion products were partially purified by lysis of the bacteria and then further purified by SDS continuous-flow gel electrophoresis. The proteins were precipitated by acetone and then resuspended in RPMI 1640 (Life Technologies) for use in proliferation assays.

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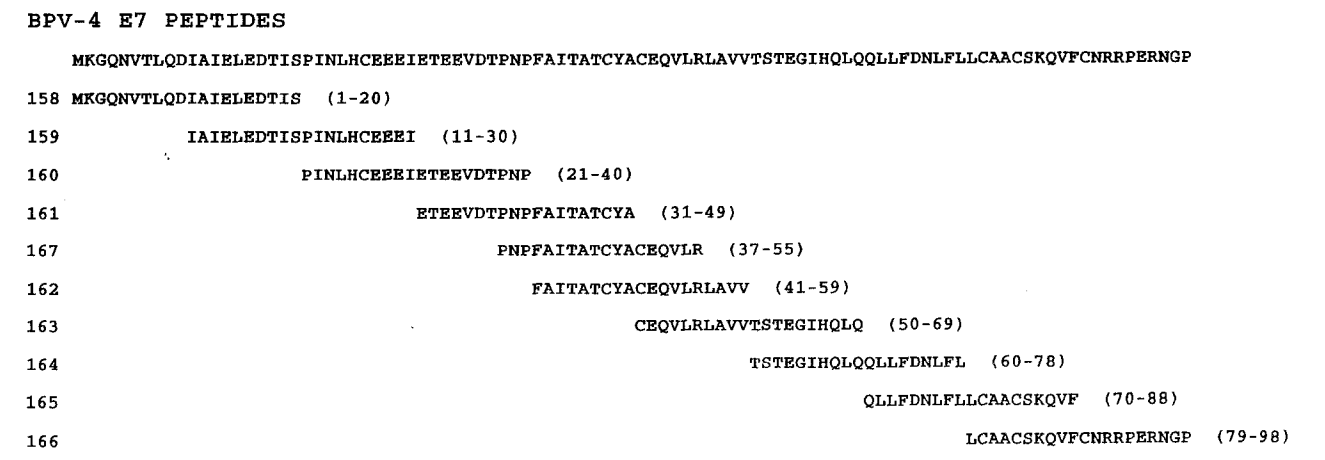

FIG. 1. Overlapping BPV-4 E7 synthetic peptides. Amino acid limits of the peptides are shown on the right and the number of the corresponding peptide on the left for ease of reference.

Vaccination

Calves approximately 12 weeks of age were divided into two groups, group 1 with 11 animals (Nos. 1–11) and group 2 with 8 animals (Nos. 12–19; N.B. animals 12, 15, and 16 are the same as 25, 26, and 27 in Chandrachud *et al.*, 1994). Each animal in group 1 was given two vaccinations of 1 mg βGal–E7 in Freund's incomplete adjuvant 4 weeks apart and then 2 weeks after the second vaccination, animals in both groups were challenged in the palate with 10^{11} particles of BPV-4.

Proliferation assays

Fifty milliliters of blood was removed from each animal at 3- or 4-week intervals throughout the trial. Blood was separated on lymphoprep (Nycomed UK) and peripheral blood mononuclear cells (PBM) removed from the interface. PBM were stored in liquid nitrogen. Proliferation assays were performed, with either fresh or frozen cells, in 96-well flat-bottomed plates. PBM at 3×10^5 cells/well in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 50 μM mercaptoethanol were cultured in medium alone or in the presence of 5 μg βGal or βGal–E7. Cells were cultured for a total of 72 hr with 1 μCi/well methyl-[^{3}H]thymidine for the last 6 hr. Harvesting was by an automatic cell harvester and incorporation was measured by a beta plate counter. Each assay was performed in triplicate. The results are presented as a stimulation index (SI); the incorporation of [^{3}H]thymidine in cells exposed to βGal was deducted from that of cells exposed to βGal–E7 to give the incorporation specific for E7: SI = cpm(βGal–E7) − cpm (βGal)/cpm (no antigen). A SI $\geq$ 2 was considered positive.

Peptides

Peptides 19 or 20 amino acids long overlapping by 10 amino acids corresponding to the entire E7 protein were synthesised and were designated 158–167 (Fig. 1) (Biomac, Ltd., Department of Biochemistry, University of Glasgow). Individual peptides were used in proliferation assays to determine the T cell epitopes of the E7 protein.

Proliferation to peptides

PBM that responded to E7 in proliferation assays were cultured at 1.5×10^6 cells/ml in 24-well tissue culture plates for 7 days in the presence of 25 μg/ml of either βGal–E7 or GST–E7 followed by 7 days with 20 units/ml recombinant human interleukin 2. Proliferation assays were carried out in 96-well flat-bottomed plates. Each well contained 1×10^5 cells after culture, 4 μg of peptide, and 2×10^5 autologous antigen presenting cells after irradiation at 30 Gy. Proliferation assays were for a total of 72 hr with methyl-[^{3}H]thymidine present for the last 6 hr. Cells were harvested as described above. Results are presented as a stimulation index: SI = cpm (peptide)/cpm (no peptide). A SI $\geq$ 2 was considered positive.

RESULTS

E7 promotes early regression of papillomas

Immunisation of cattle with βGal E7 retarded the full development of papillomas and promoted their early regression (Campo *et al.*, 1993). Vaccinated cattle did not develop papillomas beyond stage 2 (papillomas < 2 mm) compared to nonvaccinated cattle which developed stage 3 papillomas (> 2 mm). Stage 2 papillomas in the vaccinated group started to decline in numbers 13 weeks after virus challenge and the animals were papilloma free by 25 weeks postchallenge. In the control group stage 2 papillomas progressed to stage 3 papillomas and regression did not start until 25 weeks after challenge.

T cell response to E7

In vitro proliferation of PBM in response to E7 from both vaccinated and control cattle was determined at 3-

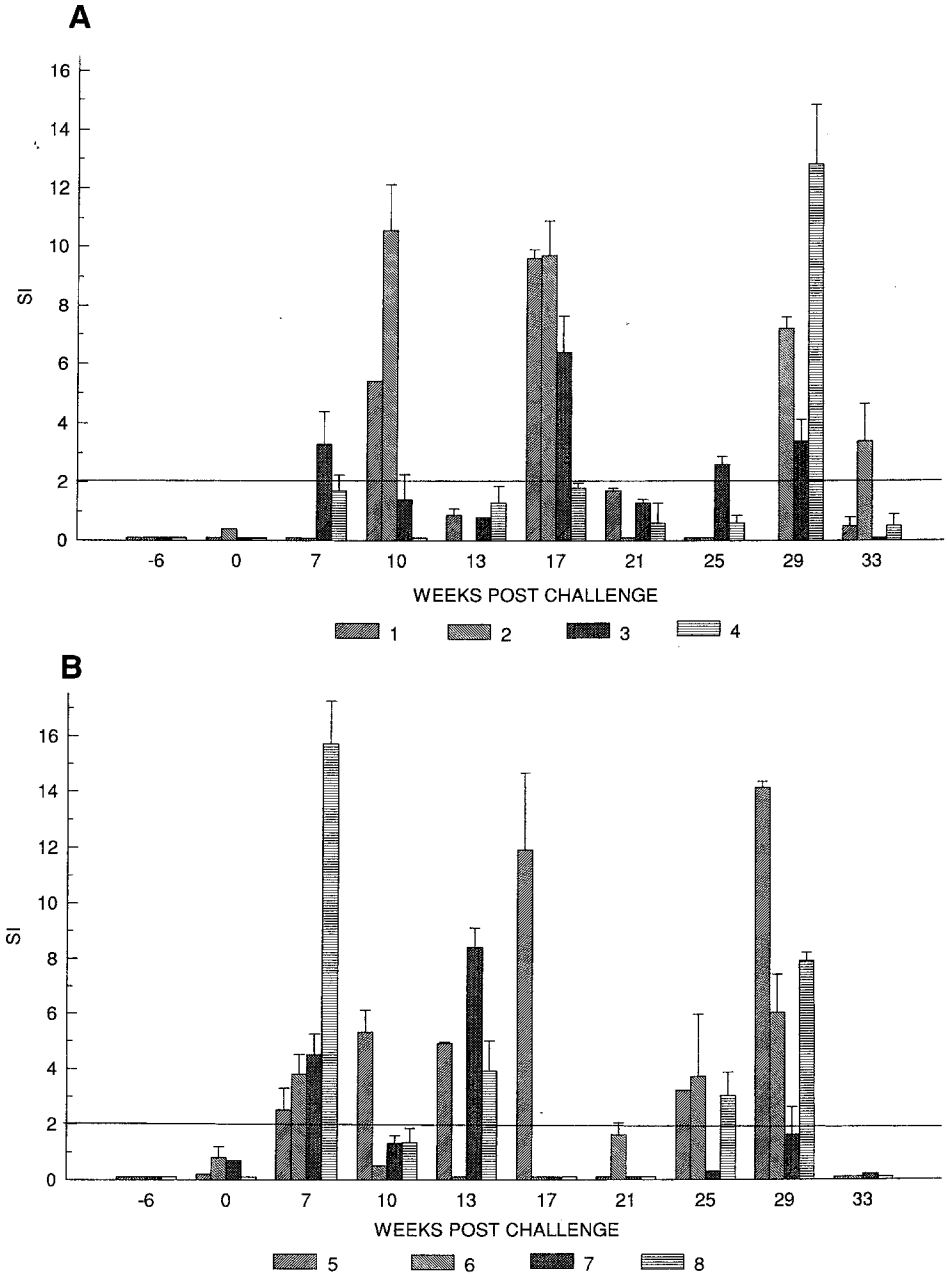

FIG. 2. Proliferative response of bovine PBM to E7 protein from E7-vaccinated animals (A, B, and C) and control animals (D) before and after challenge with BPV-4. PBM were stimulated *in vitro* with 5 μg βGal, 5 μg βGal–E7, or no antigen; each condition was performed in triplicate. Results are presented as stimulation indices and are specific responses to E7. SI = cpm βGal–E7 – cpm βGal/cpm (no antigen). The experimental number of each animal is next to the symbol box. The line represents SI = 2 and anything above this is considered positive. Error bars are not presented for responses at baseline levels.

to 4-week intervals throughout the vaccination trial before and after challenge (Fig. 2), to determine the T cell response to E7 at each stage of papilloma development and regression. PBM that were assayed more than once gave comparable and reproducible responses to E7. T cells activated by antigen *in vivo* can respond to the antigen *in vitro* and proliferate. PBM from all animals did not respond to E7 before vaccination (−6 weeks). PBM from animals in the vaccinated group proliferated to E7 between 7 and 17 weeks after challenge (Figs. 2A, 2B, and 2C). In this group the number of stage 2 papillomas had declined by 13 weeks after challenge (Campo *et al.*, 1993) and therefore E7-specific T cells were detected before and during the period of regression. There were few animals whose T cells gave stimulation indices above 2 at 21 and 25 weeks after challenge. At 29 weeks E7-specific T cells could be detected in 9 animals but only in 3 animals by 33 weeks. There appeared to be a biphasic response in most vaccinated animals over the course of the infection peaking between 7 and 17 weeks

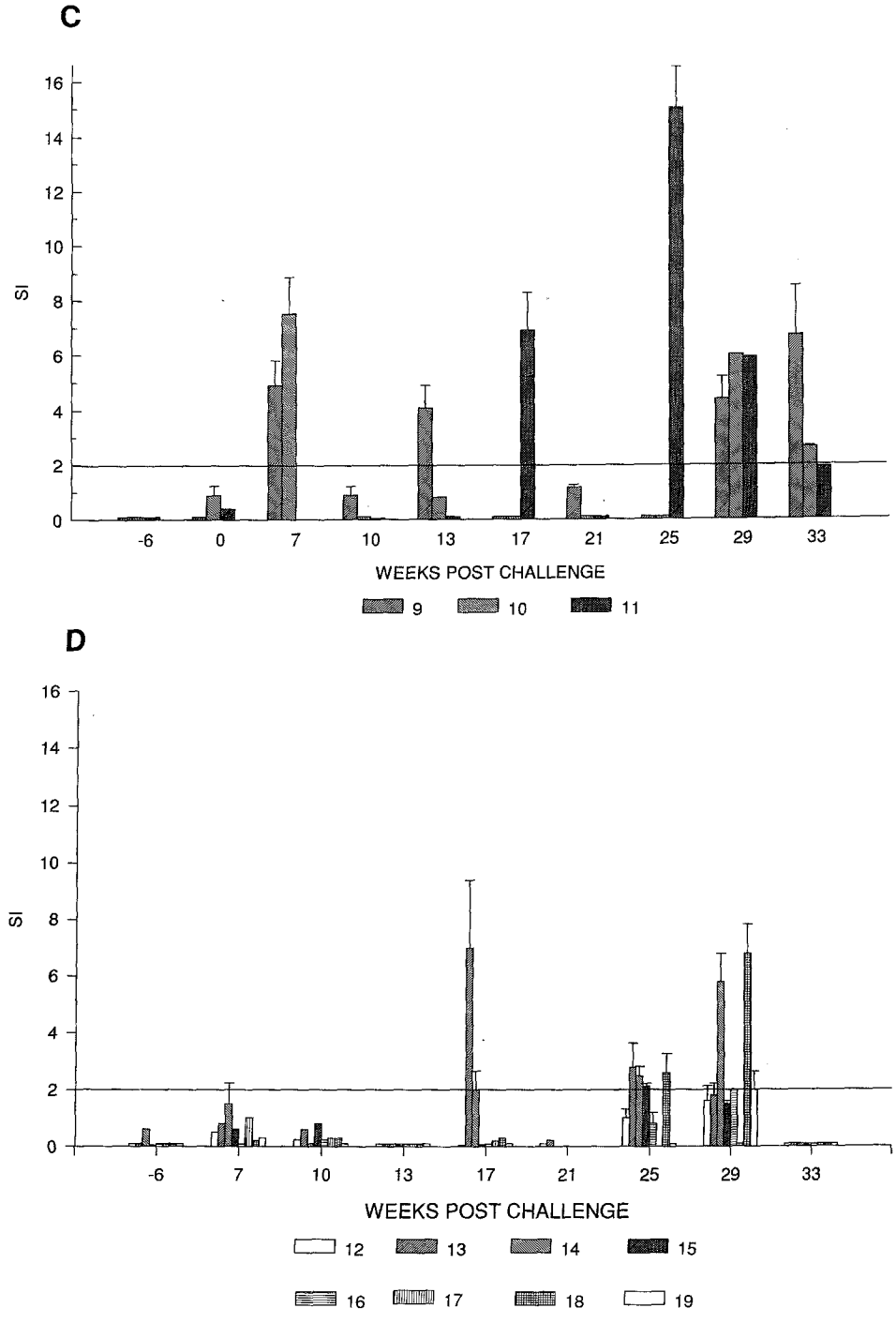

FIG. 2—*Continued*

and 29 and 33 weeks. Individual animals varied in the timing and magnitude of their response to E7. PBM from animals in the control group showed little response to E7 at all time points (Fig. 2D). E7-specific T cells could be detected in animal 13 at 17 and 25 weeks postchallenge and a further 2 animals, 14 and 18, responded at 25 and 29 weeks (Fig. 2D). The poor response to E7 in the control group at all the time points indicates that viral E7 is poorly presented to T cells *in vivo,* at least until the later stages of papilloma development. These results are consistent with the low levels of E7-specific antibodies in control animals and high levels in vaccinated animals (Chandrachud *et al.,* 1994).

T cell epitopes

Helper T cells recognise short linear peptides, approximately 8–10 amino acids in length, presented on the surface of antigen-presenting cells in the groove of MHC class II molecules. To identify the immunodominant T

TABLE 1

T CELL EPITOPES IN BPV-4 E7

Experimental animal	Weeks postchallenge	T1(aa 31–59)	T2(aa 70–88)	T3(aa 21–40)
Vaccinated				
2	10, 17	+	–	–
3	17	+	+	–
5	17	+	+	+
6	7, 25, 29	–	–	–
7	10, 13	–	–	–
8	29	+	–	–
9	29, 33	–	–	–
Total		4/7	2/7	1/7
Controls				
13	17, 25	–	–	–
14	25, 29	–	–	–
Total		0/2	0/2	0/2

cell epitopes in E7, peptides were synthesised that covered the entire E7 ORF (Fig. 1). Peptides were 19 or 20 amino acids in length overlapping by 10 amino acids. PBM from frozen stocks of 7 vaccinated animals at the peak of their response to E7 in proliferation assays (Table 1) were stimulated *in vitro* with E7 for 7 days and then a further 7 days with IL-2 to increase the population of E7-specific cells. The response to each of the peptides was then measured. While the response of individual animals was reproducible (Fig. 3) it varied between animals and was generally low, seldom achieving twice the control value even when the stimulation by E7 protein was high. PBM from 4 animals were positive to peptides covering the region aa 31–59. PBM from animals 2 and 8 were positive to peptide 167 (aa 37–55); PBM from animals 3 and 5 were positive to peptide 161 (aa 31–49) and from animal 5 to peptide 162 (aa 41–59). The region aa 31–59 recognised by the majority of animals was defined as T1. Two animals were positive to other peptides but these were not dominant to the group and contain therefore minor epitopes. PBM from animal 5 responded strongly to peptide 160 (aa 21–40), which has a 10-amino-acid overlap with peptide 161 (aa 31–49), and 2 animals, 3 and 5, responded to peptide 165 (aa 70–88). The two minor epitopes, amino acids 70–88 and 21–40, have been defined as T2 and T3, respectively. Two control (nonvaccinated) animals, 13 and 14, that had a SI > 2 to E7, were analysed for their response to the peptides at Weeks 17 and 25 for animal 13 and Weeks 25 and 29 for animal 14. Individual peptides did not stimulate PBM from either animal above twice the control. The response of individual animals to the three epitopes is summarised in Table 1 and the epitopes are highlighted in Fig. 4.

DISCUSSION

We have previously shown that vaccination of cattle with BPV-4 E7 before challenge with BPV-4 has a therapeutic effect, promoting early regression of papillomas (Campo *et al.*, 1993). It has been well documented that a vaccine should elicit both a humoral and a cell-mediated immune response (Tindle *et al.*, 1991). BPV-4 E7 appears to fulfill these requirements; high levels of E7-specific antibodies have been detected in the sera of cattle after vaccination (Chandrachud *et al.*, 1994) and this paper shows that E7-specific T cells can be detected in the peripheral blood of cattle after vaccination at significantly higher levels than in nonvaccinated animals. Both the T cell and antibody responses to E7 are statistically different between the vaccinated and nonvaccinated groups (P-value < 0.001), confirming that vaccination with E7 significantly enhances both the cell-mediated and humoral response to viral E7. The nature of the T cell response to E7 over time is difficult to assess due to the differences in timing and magnitude of the response in individual animals but this variability would be expected in an outbred population. The peak in T cell activity between 7 and 17 weeks corresponds to the time of regression of stage 2 papillomas in vaccinated animals. This suggests that E7-specific T cells may play a role in vaccine induced early regression of papillomas but the mechanism of regression remains for the moment unknown. There was no unequivocal correlation between strength and timing of a proliferative response and the time of regression in individual animals. As the vaccine was administered as exogenous protein, it is likely that the activated T cells detected in the proliferation assay are helper T cells stimulated in an MHC class II restricted manner, although other cell types cannot be ruled out. Cattle have a high level of $CD2^-$ $CD4^-$ $CD8^-$ T cells with $\gamma\delta$ T cell receptors, associated with protection of epithelial surfaces (Hein and Mackay, 1991); these cells could contribute to the response to E7 and papilloma regression.

In nonvaccinated cattle there is a weak T cell response to E7, which is consistent with the low levels of E7-specific antibodies detected in the animals (Chandrachud *et al.*, 1994). These observations show that viral E7 is poorly presented to the immune system, at least until the later stages of papilloma development. BPV-4 infects keratinocytes which do not possess MHC class II and, as infection does not kill the cell, presentation to T helper cells may be limited.

The bovine model demonstrates that vaccination with BPV-4 E7 induces regression of BPV-4 papillomas and this is accompanied by a significant humoral and cell-mediated response to E7. Studies with HPV-16 E7 in rodent models have shown that E7 acts as a tumour rejection antigen (Meneguzzi *et al.*, 1991; Chen *et al.*, 1991) and induces a delayed-type hypersensitivity response (McLean *et al.*, 1993). Both antibodies and T cells specific for HPV-16 E7 have been detected in mice (Commerford *et al.*, 1991; Shepherd *et al.*, 1992; Tindle *et al.*, 1991) and HPV-16 E7-specific T cells have been reported in seropositive patients (Altmann *et al.*, 1992). Taken to-

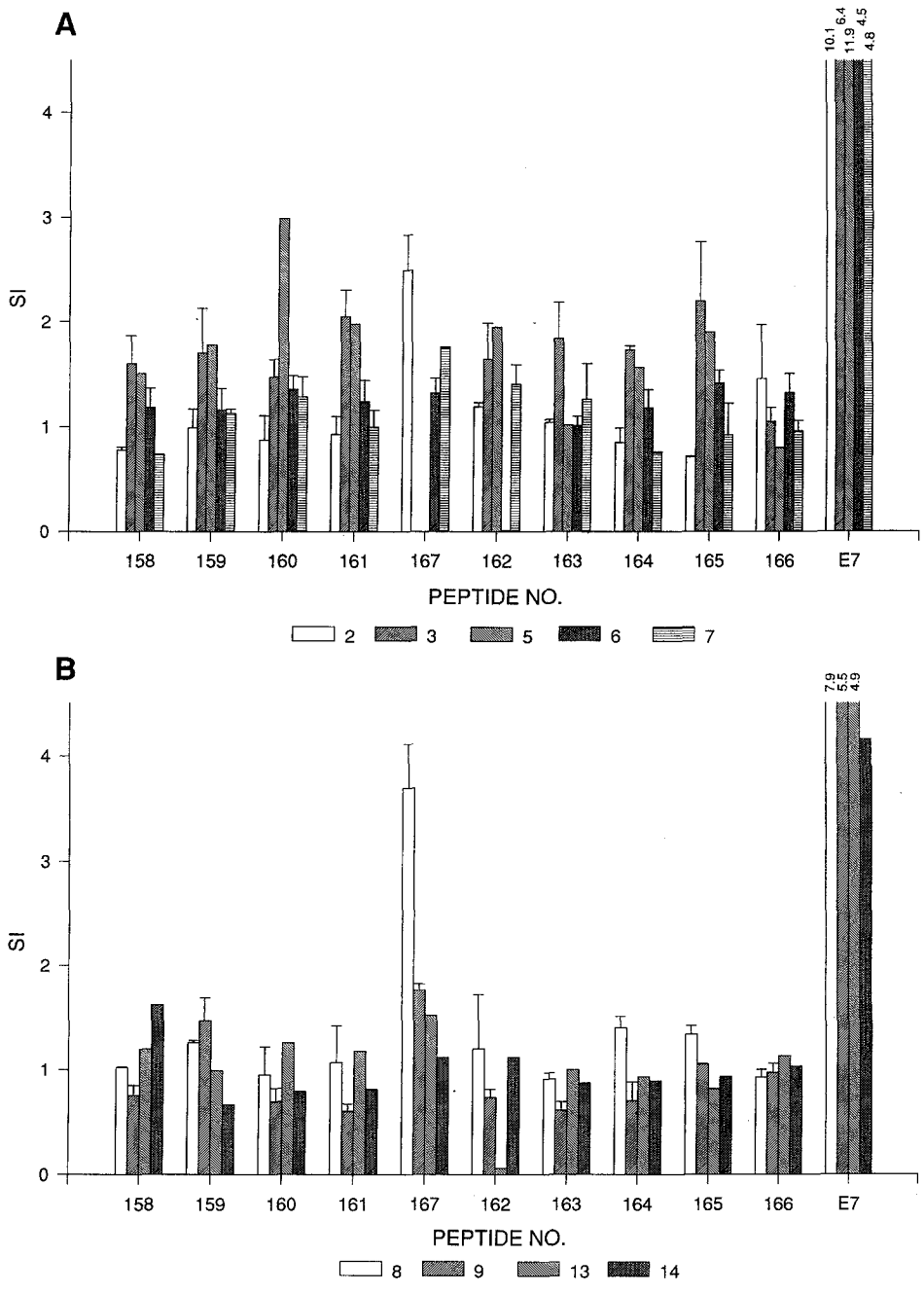

FIG. 3. T cell proliferation assays of E7 responder cattle to peptides. PBM from cattle responding to E7 in proliferation assays were stimulated *in vitro* with E7 and then used in proliferation assays with peptides (158–167) as antigen at 4 μg/well in the presence of autologous irradiated PBM as antigen-presenting cells. Assays were in triplicate and [^{3}H]thymidine incorporation was measured and presented as stimulation indices. The experimental number of each animal is next to the symbol box. The response of PBM to E7 is represented on the graph and SI are written above each bar. SI = cpm peptides/cpm (no antigen).

gether, our results and those from other laboratories strongly suggest that E7 is a potential candidate for therapeutic vaccination against papillomavirus infection in humans.

From peptide mapping studies BPV-4 E7 appears to contain at least one immunodominant T cell epitope (T1) recognised by the majority of animals and two minor epitopes (T2 and T3) recognised by only one or two animals. T1, because of its length, 29 amino acids, may contain more than one epitope. No single peptide was recognised by all the animals; however, variability in response to the peptides is not surprising in an outbred population where there are differing MHC alleles. T cell epitopes have been mapped on HPV-16 E7 along 75% of the protein length (Commerford *et al.*, 1991; Shepherd *et al.*, 1992; Tindle *et al.*, 1991; Altmann *et al.*, 1992)

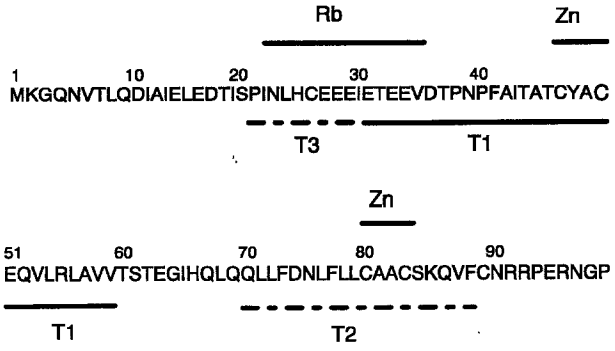

FIG. 4. Amino acid sequence of BPV-4 E7 with the T cell epitopes and functional domains highlighted. Solid lines above the sequence represent functional domains and below the sequence major T cell epitopes; dashed lines below the sequence represent minor T cell epitopes.

and differences in response to E7 peptides have been reported in different strains of mice (Shepherd *et al.*, 1992). The T cell epitope defined in BPV-4 E7 as being immunodominant covers part of the putative p105Rb binding domain and the putative Zn^{2+} binding domain (Fig. 4), areas which have high amino acid homology with HPV-16 E7 (Jaggar *et al.*, 1990; Jackson *et al.*, 1991). Whether BPV-4 E7 and HPV-16 E7 have similar immunological characteristics remains to be determined, but the results with BPV-4 E7 give hope that HPV-16 E7 may have a similar effect in inducing papilloma regression in humans.

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